Hydroxocobalamin. I. Blood Levels and Urinary Excretion of Vitamin B_{12} in Man After a Single Parenteral Dose of Aqueous Hydroxocobalamin, Aqueous Cyanocobalamin and Cyanocobalamin Zinc-Tannate Complex

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IN GENERAL, the treatment of vitamin B_{12} deficiencies presents few difficulties. In B_{12} deficiency anemias, and later for the maintenance of the body's B_{12} stores, the daily requirements of vitamin B_{12} necessary for hematopoietic response can easily be met by the usual schedules of parenteral treatment. In states of advanced vitamin B_{12} depletion, the deficit of B_{12} may amount to 3000 μg., or more, of B_{12}.[1] To replenish this deficiency, large parenteral doses of cyanocobalamin are frequently used. This unavoidably results in massive urinary losses amounting, on the average, to about 75 per cent of a 200 μg. dose, and to as much as 86 per cent of a 1000 μg. dose.[2]

Attempts have consequently been made to develop a long-acting B_{12} which can be retained in the body more efficiently than cyanocobalamin.[3] This has been tried by combining cyanocobalamin with sesame oil and aluminum monostearate,[4,5] or by making it into a zinc-tannate complex.[6-8] How much of the cyanocobalamin in these complexes is fully available to the body's metabolic processes as vitamin B_{12}, however, has yet to be established.

In our search for a long-acting B_{12} preparation, cobalamin preparations in various media and in various modifications were screened and their turnover compared in vivo with that of aqueous cyanocobalamin and a cyanocobalamin zinc-tannate complex. The following preparations were studied: cyanocobalamin in oil gel; cobalamin-glycine and cobalamin-glutathione complexes; hydroxocobalamin in aqueous solution, in oil gel, in gelatine medium and in combination with a cyanocobalamin zinc-tannate complex. In these studies, which will be reported elsewhere,[9] only an aqueous solution of hydroxocobalamin* was found to possess the features sought for in a long-acting B_{12}. Hydroxocobalamin (formerly known as "vitamin B_{12a}" and aquocobalamin ("vitamin B_{12b}") are known to show potent hematopoietic activity at a dose of 1 to 2 μg. daily in man[10-15] (table 1) and metabolic activity in microorganisms,[16,17] especially in the presence of reducing agents.[17]

We therefore made a detailed study of the applicability of aqueous hy-

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Table 1.—*Hematopoietic Activity of Hydroxocobalamin or Aquocobalamin*

Authors	Year	No. of cases	Vitamin B_{12a} or B_{12b} treatment: Dose i.m.	Frequency of inject.	Duration	Hematopoietic activity
West[11]	1949	1	25 μg.	Single		Equivalent to 30% of maximal response.
Lichtman et al.[12]	1949	3	1–2 μg.	Daily	3 weeks	Effective at an optimal dose of 1–2 μg. daily.
Spies et al.[13]	1950	4	10 μg.	Single		As effective as cyanocobalamin.
Schilling et al.[14]	1951	7	1–4 μg.	Daily	13–16 days	As effective as cyanocobalamin at a dose of 1–2 μg. daily.
Meyer et al.[15]	1953	9	1–4 μg.	Daily	5–30 weeks	Effective at a dose of 2 μg. daily.

droxocobalamin as a long-acting vitamin B_{12} in man, the report of which follows. A preliminary report of this work was read at the 7th International Congress of Hematology in Tokyo, Sept. 4–10, 1960,[18] and a short abstract of some of these findings has been published elsewhere.[19]

Materials and Methods

Fifty-three patients in all were studied, all with normal or low B_{12} blood levels and no evidence of circulatory or renal failure, as shown by normal urinalysis, phenolsulphonphthalein and BUN tests.

Seventeen subjects received hydroxocobalamin in aqueous solution; 19 received cyanocobalamin in aqueous solution, and 17 received cyanocobalamin zinc-tannate complex. These materials were injected in a single dose of 500 or 1000 μg. by means of disposable syringes and needles. Blood was withdrawn (again, with disposable syringes and needles) just prior to injection and thereafter at the 5th, 24th, 48th and 72nd hours, and on the 7th, 14th, 21st and 28th days after injection. On collection, the red cells were immediately separated from the blood serum to prevent any B_{12} exchanges. Five specimens of urine, covering the 0–6, 6–12, 12–24, 24–48 and 48–72-hour periods, were collected during the 3 days after injection. In addition, 24-hour urine specimens were obtained on the 7th, 14th, 21st and 28th days following injection. All glassware had been pretreated to prevent contamination and was used only once. Determination of total vitamin B_{12} values in blood serum and urine was made by *Lactobacillus leichmannii* assay.[20]

Results

Microbiological Assays of Blood Serum for Vitamin B_{12}

The results are summarized in table 2 and in figures 1 and 2.* A single i.m. injection of 1000 μg. of hydroxocobalamin resulted in an average 96-fold rise in the vitamin B_{12} blood serum levels by the 5th hour, a 52-fold rise by the 24th hour, a 23-fold rise by the 48th hour and a 10-fold rise by the 72nd hour.

*The first three cases treated with non-stabilized hydroxocobalamin have been omitted from table 2 and figures 1 and 2.

Table 2.—*Comparison of Vitamin B_{12} Blood Serum Levels After I.M. Injection of Aqueous Cyanocobalamin and Hydroxocobalamin at Doses of 500 and 1000 μg.*

Time	Vitamin B_{12} blood serum levels mμg./ml. (means with standard deviations)		Statistical evaluation of the difference*		
	Cyanocobalamin (19 cases)	Hydroxocobalamin (14 cases)	t	n	p
	1000 μg. i.m.				
Before injection	.50 ± .18	.60 ± .15	1.25	20	Not signif.
5 hr. after injection	14.00 ± 4.96	57.72 ± 10.15	9.94	17	<.001
24 hr. after injection	2.44 ± 1.56	31.66 ± 9.47	7.49	16	<.001
48 hr. after injection	1.97 ± 2.02	14.03 ± 3.09	8.55	15	<.001
72 hr. after injection	1.19 ± .49	6.22 ± 2.35	5.13	12	<.001
1 wk. after injection	.80 ± .32	3.29 ± .55	10.37	14	<.001
2 wk. after injection	.62 ± .26	1.51 ± .30	5.56	12	<.001
3 wk. after injection	.63 ± .26	1.27 ± .22	4.57	12	<.001
4 wk. after injection	.72 ± .30	1.49 ± 1.04	1.75	12	Not signif.
	500 μg. i.m.				
Before injection	.43 ± .20	.35 ± .21	.22	12	Not signif.
5 hr. after injection	10.68 ± 3.32	18.88 ± 6.20	2.85	12	<.02
24 hr. after injection	2.12 ± .96	9.61 ± 3.09	5.67	12	<.001
72 hr. after injection	.94 ± .39	2.30 ± .43	1.81	12	Not signif.
1 wk. after injection	.86 ± .16	.95 ± .26	.69	10	Not signif.
2 wk. after injection	.44 ± .09	.71 ± .22	2.70	10	<.05
3 wk. after injection	.38 ± .10	.59 ± .22	2.10	10	<.05
4 wk. after injection	.25 ± 0	.56 ± .23	3.10	10	<.02

*$t = D/E_D$, test of significance of difference of means, where D = difference of means, and E_D = standard error of difference of means. n = number of degrees of freedom available for estimation of error, calculated as sum of tests in each group minus 2. p = probability value of significance of difference of means, calculated from Table III of Fisher-Yates Statistical Tables. Differences showing values of p below 0.05 were accepted as statistically significant, those below 0.02 as highly significant.

For a 3-week period the blood serum levels of B_{12} were significantly higher statistically after i.m. injection of a single dose of 1000 μg. of hydroxocobalamin than after an identical dose of cyanocobalamin. While the initial blood levels did not show any statistically significant difference, the mean levels were 1.8 and 4.1 times higher, respectively, 5 hours after injection of 500 or 1000 μg. of hydroxocobalamin, 4.6 and 12.8 times higher by the 24th hour, 2.4 and 5.2 times higher by the 72nd hour, and 1.6 and 2.4 times higher by the 2nd through the 4th week than after administration of identical doses of cyanocobalamin. These differences were highly significant statistically.

Compared with identical doses of cyanocobalamin zinc-tannate complex (fig. 1), blood levels following i.m. administration of 1000 μg. hydroxocobalamin were 15.6 times higher by the 5th hour, 10.8 times higher by the 24th hour and 3.6 times higher by the 48th hour. These differences were highly significant statistically. Similar blood level differences were also observed during the first 24 hours after injection of 500 μg. of hydroxocobalamin and zinc-tannate complex (fig. 2). One to 3 weeks after injection, B_{12} blood levels were slightly higher after cyanocobalamin zinc-tannate than after hydroxocobalamin.

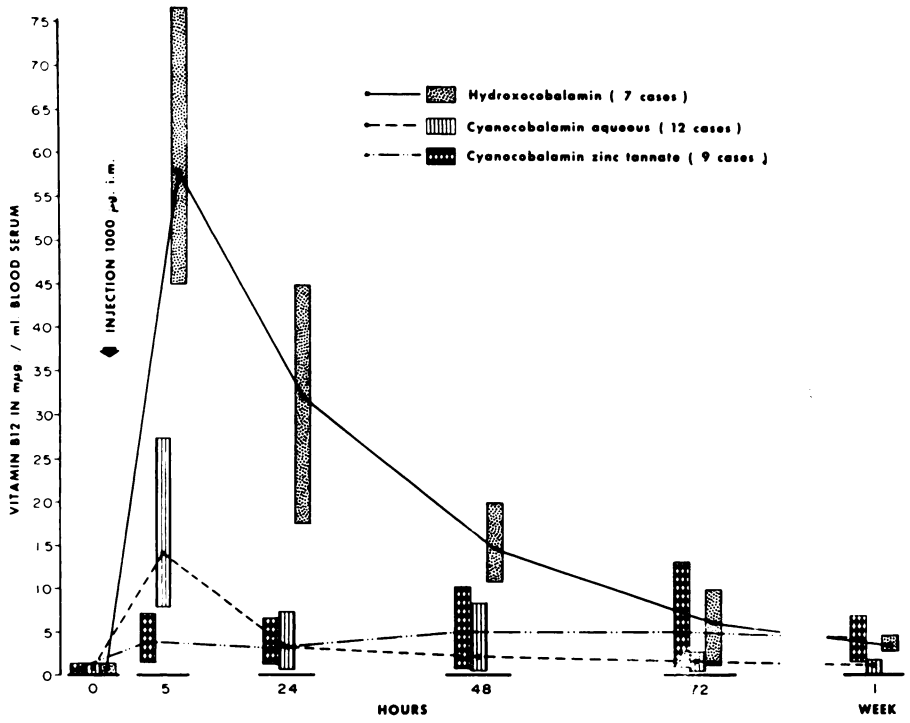

Fig. 1.—Vitamin B_{12} blood serum levels following single i.m. injection of 1000 μg. hydroxocobalamin, cyanocobalamin aqueous and cyanocobalamin zinc-tannate complex.

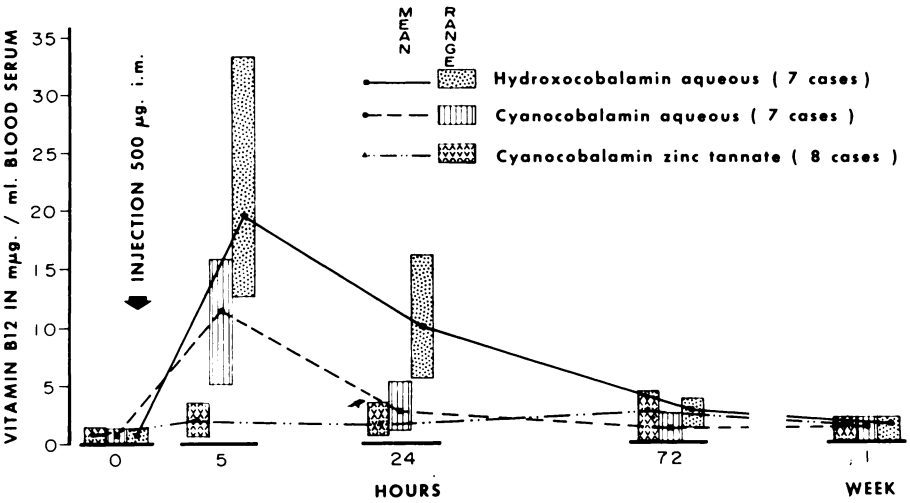

Fig. 2.—Vitamin B_{12} blood serum levels following single i.m. injection of 500 μg. hydroxocobalamin, cyanocobalamin aqueous and cyanocobalamin zinc-tannate complex.

On statistical evaluation, however, the latter differences were found to be insignificant.

Microbiological Assays of Urinary Excretion of Vitamin B_{12}

The data obtained following administration of cyanocobalamin and hydroxocobalamin are shown in tables 3 and 4 and figures 3–5.

Aqueous hydroxocobalamin was excreted in the urine at a much less rapid rate than aqueous cyanocobalamin. The difference in quantity excreted is due mainly to the slower rate of urinary spilling of hydroxocobalamin during the first 24 hours as compared with that of cyanocobalamin. After single i.m. injections of 500 or 1000 μg. of hydroxocobalamin, only 16 per cent and 27 per cent, respectively, are lost in the 72-hour urines, while corresponding losses of cyanocobalamin, at equal doses and time intervals, amount to 60 per cent and 69 per cent, respectively, of the amount injected. During the first 72 hours, urinary losses of B_{12} following administration of similar doses of cyanocobalamin zinc-tannate complex amount to only 14 per cent and 20 per cent, respectively, of the injected material. The body's retention of cyanocobalamin under these circumstances is not associated with high B_{12} blood levels, however, as in the case of hydroxocobalamin.

Table 3.—*Comparison of Urinary Output of Vitamin B_{12} After I.M. Injection of Aqueous Cyanocobalamin and Hydroxocobalamin at Doses of 500 and 1000 μg.*

Time	Urinary output (μg.)		Statistical evaluation of the difference*		
	Cyanocobalamin (13 cases)	Hydroxocobalamin (13 cases)	t	n	p
	1000 μg. i.m.				
0–24 hours	601.9 ± 182.2	259.9 ± 66.6	4.17	12	<.01
24–72 hours	3.6 ± 6.9	10.3 ± 3.8	2.09	12	Not signif.
0–72 hours	605.5 ± 186.8	270.3 ± 68.9	4.13	12	<.01
	500 μg. i.m.				
0–24 hours	341.2 ± 70.0	77.6 ± 19.0	8.85	10	<.001
24–72 hours	5.6 ± 10.3	2.8 ± 0.9	0.61	10	Not signif.
0–72 hours	346.8 ± 80.1	81.2 ± 20.9	7.18	10	<.001

*t, n and p = as in table 1.

Table 4.—*24-Hour Urinary Output of Vitamin B_{12} in 7 Individuals, 1, 2, 3 and 4 Weeks After I.M. Administration of 1000 μg. Hydroxocobalamin*

Case no.	24-Hour output of vitamin B_{12}, in μg., on			
	7th day	14th day	21st day	28th day
1	0.48	0.06	0.07	0.01
2	0.36	0.26	0.06	0.06
3	0.15	0.14	0.02	
4	0.47	0.26	0.05	0.06
5	0.53	0.10	0.05	0.01
6	0.09	0.02	0.02	0.01
7	0.31	0.06	0.10	0.04
Mean	0.34	0.13	0.04	0.03

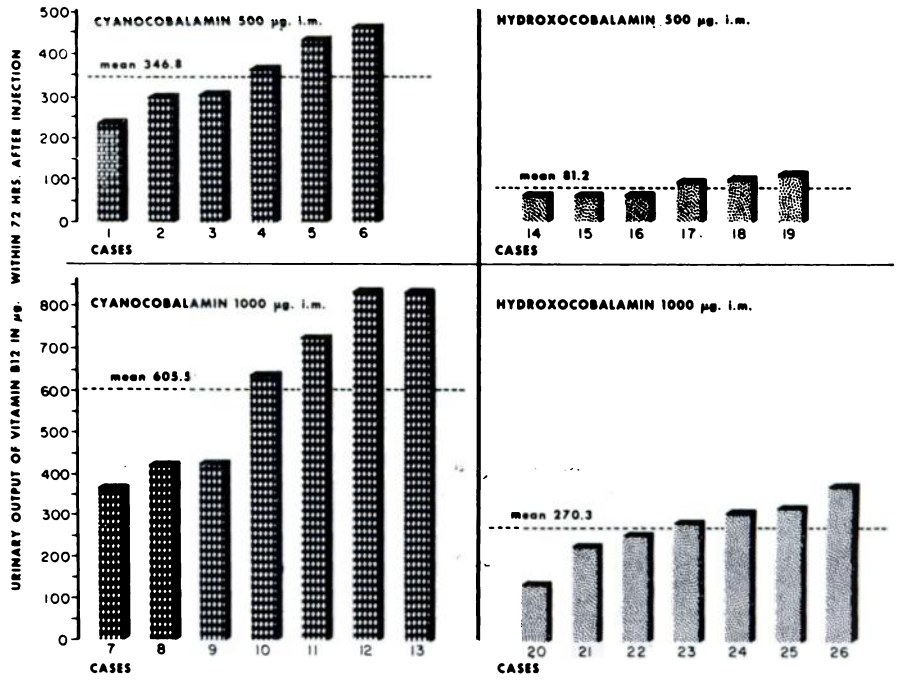

Fig. 3.—72-Hour urinary output of vitamin B_{12} following i.m. injection of cyanocobalamin and hydroxocobalamin at 500 μg. and 1000 μg. doses.

Urinary elimination of hydroxocobalamin continues to be exceedingly slow during the weeks that follow. The 24-hour output of vitamin B_{12} was measured in 7 individuals on the 7th, 14th, 21st and 28th days after i.m. administration of 1000 μg. hydroxocobalamin. These data, listed in table 4, show that there is a further gradual decrease in the daily output of hydroxocobalamin. The amounts excreted daily on the 7th to the 28th days after i.m. injection of 1000 μg. of hydroxocobalamin are exceedingly small, amounting to only a small fraction of 1 μg. This indicates that the urinary excretion of hydroxocobalamin is negligible in the later weeks following injection.

Discussion

The results of these studies give evidence of a slower rate of urinary excretion of hydroxocobalamin, as compared to that of cyanocobalamin, and of its ability to build up consistently higher and more prolonged vitamin B_{12} levels in the blood. These findings suggest the possible clinical applicability of hydroxocobalamin as a long-acting vitamin B_{12}. They also tend to indicate that the metabolic turnover of hydroxocobalamin is somewhat slower than that of cyanocobalamin and that the biological half-life of hydroxocobalamin may be longer than the average one year of cyanocobalamin.[21-25] Further study will be required to determine whether the metabolic pathways of these two substances are similar.

The underlying factors in what is probably a slower metabolic turnover of

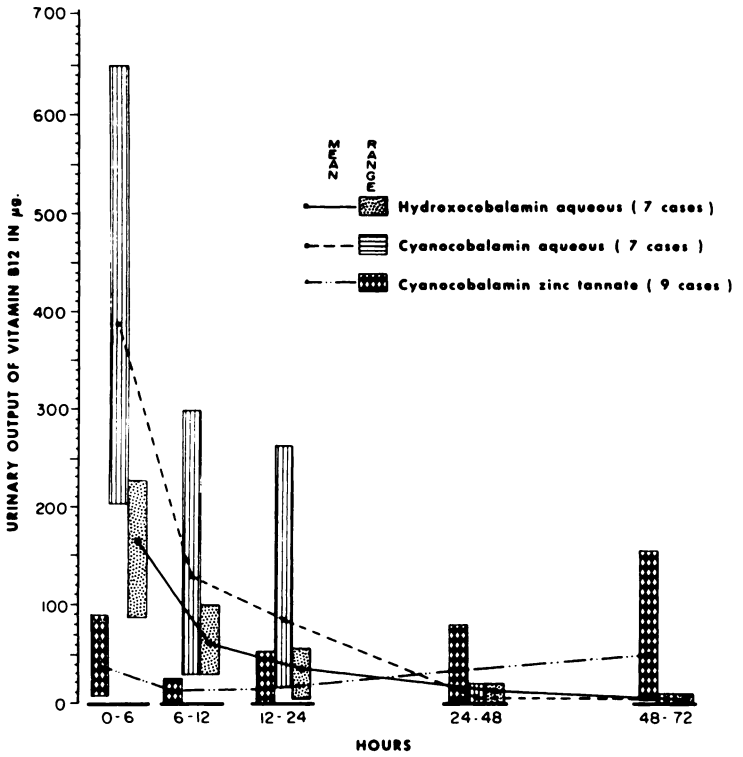

Fig. 4.—Comparison of total vitamin B_{12} excreted in the urine after i.m. injection of a single 1000 μg. dose of aqueous hydroxocobalamin, aqueous cyanocobalamin and cyanocobalamin zinc-tannate complex.

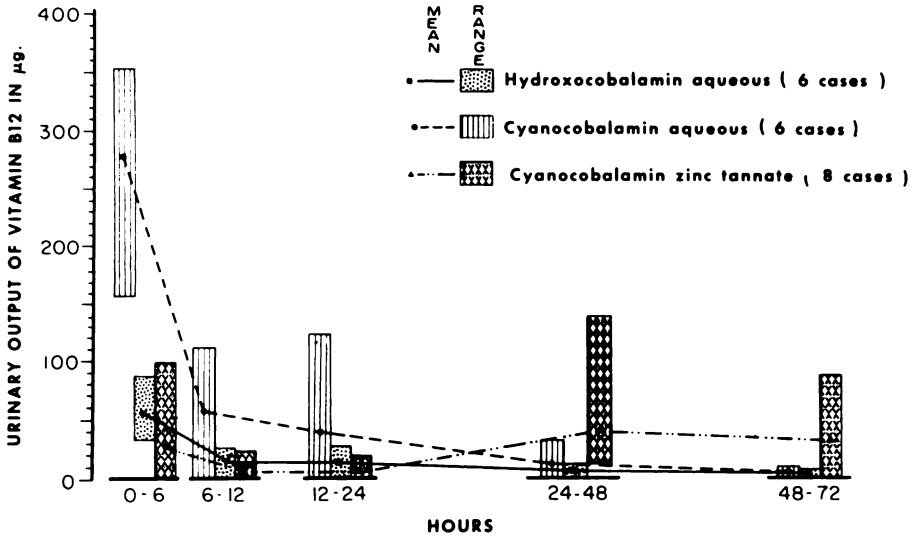

Fig. 5.—Comparison of total vitamin B_{12} excreted in the urine after i.m. injection of a single 500 μg. dose of aqueous hydroxocobalamin, aqueous cyanocobalamin and cyanocobalamin zinc-tannate complex.

hydroxocobalamin are its greater reactivity and its ability to form stronger bonds with the body's B_{12} binders, including the blood proteins.[26-28] These bonds may account for the delay in the disappearance of hydroxocobalamin from the blood.

The slower urinary elimination of hydroxocobalamin may also be due to the stronger bonds it forms with the tissues and blood proteins. Alternate possibilities are (1) decreased urinary clearance of hydroxocobalamin by the kidneys or (2) its excessive fecal excretion as a result of the enterohepatic circulation of B_{12}. The latter possibility seems unrealistic, however, since the biliary excretion of Co^{58}-labeled cyanocobalamin, over a 6-day period, after i.m. administration at a dose of 225 μg. to an individual with common duct drainage and normal liver amounted to only 1.4 μg., i.e. only 0.6 per cent of the dose injected.[29] There is no reason why hydroxocobalamin, which forms stronger bonds with the blood proteins than cyanocobalamin, should be more easily detached from these bonds and more rapidly eliminated into the intestine. More direct information, however, is needed on the enterohepatic turnover and renal clearance of hydroxocobalamin.

Summary

A single intramuscular injection of 500 or 1000 μg. of hydroxocobalamin to 17 individuals resulted in a 1.8- to 4.1-times higher mean serum vitamin B_{12} blood level, respectively, 5 hours after injection; a 4.6- and 12.8-times higher level 24 hours after injection; a 2.4- and 5.2-times higher level 72 hours after injection, and a 1.6- and 2.4-times higher level by the 2nd through the 4th week after injection than identical doses of cyanocobalamin administered to 19 individuals. The vitamin B_{12} blood levels following i.m. administration of 500 or 1000 μg. of hydroxocobalamin were significantly higher during the first 24 and 48 hours, respectively, than they were after a cyanocobalamin zinc-tannate complex given to 17 individuals at identical doses.

After a single i.m. injection of 500 or 1000 μg. of hydroxocobalamin, an average of only 16 per cent and 27 per cent, respectively, of the vitamin B_{12} was lost in the 72-hour urines, as compared to 60 per cent and 69 per cent, respectively, after identical doses of cyanocobalamin. These differences, again, were highly significant statistically.

The results of these studies give evidence of a slower rate of urinary excretion of hydroxocobalamin as compared to that of cyanocobalamin, and of its ability to build up consistently higher and more prolonged vitamin B_{12} levels in the blood.

Summario in Interlingua

Un sol injection intramuscular de 500 o 1000 μg de hydroxocobalamina a 17 subjectos resultava—in comparation con simile injectiones del mesme dose de cyanocobalamina a 19 subjectos—in valores medie del nivellos sanguinee de vitamina B_{12} que esseva superior, respectivemente, per le factores 1,8 e 4,1 al fin de 5 horas, per le factores 4,6 e 12,8 al fin de 24 horas, per le factores 2,4 e 5.2 al fin de 72 horas, e per le factores 1,6 e 2,5 durante le intervallo ab le secunde usque al fin del quarte septimana post le injection. Le nivellos

sanguinee de vitamina B_{12} post le administration intramuscular de 500 o 1000 μg de hydroxocobalamina esseva significativemente plus alte al fin de 24 e 48 horas que le correspondente nivellos in 17 subjectos tractate con doses identic de complexo de cyanocobalamina e tannato de zinc.

Post un injection intramuscular de un sol dose de 500 o 1000 μg de hydroxocobalamina, le quantitates medie de respectivemente non plus que 16 e 27 pro cento del vitamina B_{12} esseva perdite in le urina del prime 72 horas, a comparar con (respectivemente) 60 e 69 pro cento post doses identic de cyanocobalamina. Iste differentias es, de novo, statisticamente significativissime.

Le resultatos del presente studios reflecte un plus lente excretion urinari de hydroxocobalamina que de cyanocobalamina. Hydroxocobalamina es etiam plus capace que cyanocobalamina de effectuar uniformemente plus alte e plus persistentemente elevate nivellos de vitamina B_{12} in le sanguine.

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